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RESEARCH ARTICLE

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Bioactive compounds from the African medicinal plant *Cleistochlamys kirkii* as resistance modifiers in bacteria

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Cleistochlamys kirkii (Benth) Oliv. (Annonaceae) is a medicinal plant traditionally used in Mozambique to treat infectious diseases. The aim of this study was to find resistance modifiers in *C. kirkii* for Gram-positive and Gram-negative model bacterial strains. One of the most important resistance mechanisms in bacteria is the efflux pump-related multidrug resistance. Therefore, polycarpol (**1**), three *C*-benzylated flavanones (**2**–**4**), and acetylmelodorinol (**5**) were evaluated for their multidrug resistance-reverting activity on methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* and *Escherichia coli* AG100 and AG100 A strains overexpressing and lacking the AcrAB–TolC efflux pump system. The combined effects of antibiotics and compounds (**2** and **4**) were also assessed by using the checkerboard microdilution method in both *S. aureus* strains. The relative gene expression of the efflux pump genes was determined by real-time reverse transcriptase quantitative polymerase chain reaction. The inhibition of quorum sensing was also investigated. The combined effect of the antibiotics and compound **2** or **4** on the methicillin-sensitive *S. aureus* resulted in synergism. The most active compounds **2** and **4** increased the expression of the efflux pump genes. These results suggested that *C. kirkii* constituents could be effective adjuvants in the antibiotic treatment of infections.

KEYWORDS

C-benzylated flavanones, *Cleistochlamys kirkii*, efflux pump, *Escherichia coli* AG100, methicillin-resistant *Staphylococcus aureus*, quorum sensing

1 | INTRODUCTION

The increasing resistance of bacterial isolates is a high concern in the therapy of infectious diseases. The widespread and inappropriate use of antibiotics has contributed to the selection of resistant bacteria. Multidrug-resistant (MDR) bacteria show resistance against a broad range of antimicrobials, and one of the most important resistance mechanisms is the presence of multidrug-resistant efflux pumps (EPs). These membrane proteins have physiological functions, and they are involved in the extrusion of toxic substances into the environment (Aparna, Dineshkumar, Mohanalakshmi, Velmurugan, & Hopper, 2014; Webber & Piddock, 2003).

In *Staphylococcus aureus*, the most studied MDR pump is the chromosomally encoded NorA, which is a member of the major facilitator superfamily. NorA can transport hydrophilic compounds, quaternary ammonium compounds, and dyes. The MepA chromosomally encoded efflux transporter described in *S. aureus* belongs to the multidrug and toxic compound extrusion family. MepA can recognize fluoroquinolones,

glycylcyclines, dyes, and quaternary ammonium compounds (Costa, Viveiros, Amaral, & Couto, 2013).

The discovery of plant-derived antimicrobials has drawn particular attention (Balogh et al., 2014; Hintz, Matthews, & Di, 2015). Numerous phytochemicals have minimal toxic activity, and they could be used in order to overcome drug resistance in bacteria by blocking multidrug EPs (Stavri, Piddock, & Gibbons, 2007). It is important to note that EP inhibitors (EPIs) from plant sources can inhibit the activity of bacterial efflux systems; furthermore, they can potentiate the efficacy of antibiotics as well. It has been described that reserpine, isolated from the roots of *Rauwolfia vomitoria*, inhibits the Bmr EP of *Bacillus subtilis* (Klyachko, Schuldiner, & Neyfakh, 1997). Several *Berberis* species producing berberine were also found to synthesize 5′-methoxyhydnocarpin, an inhibitor of the NorA MDR pump of *S. aureus* (Stermitz, Lorenz, Tawara, Zenewicz, & Lewis, 2000). Cucurbitane-type triterpenoids, isolated from the aerial parts of the African medicinal plant *Momordica balsamina*, have shown activity against the EP systems of Gram-positive bacteria (Ramalhete, Da Cruz, et al., 2011a; Ramalhete, Lopes, et al., 2011b; Ramalhete, Spengler, et al., 2011c).

The bacterial quorum sensing (QS) is a cell-to-cell communication system that is based on chemical signals, namely, autoinducers (AIs). In Gram-negative bacteria, the most common AI is the *N*-acyl homoserine lactone (AHL; Varga et al., 2011). EPs and QS signals play an important role in the development of bacterial virulence. The QS system and the AIs are able to influence the expression of transporter genes. These EPs have the ability to transport AI molecules to the external environment of bacteria, thus facilitating cell-to-cell communication (Spengler, Kincses, Gajdács, & Amaral, 2017).

Cleistochlamys kirkii (Benth) Oliv. (Annonaceae) is an African medicinal plant traditionally used in Mozambique for the treatment of wound infections, tuberculosis, and rheumatism (Verzár & Petri, 1987).

Previously, in a research for antimicrobial compounds from African medicinal plants (Cabral et al., 2015; Kolaczkowski et al., 2010; Madureira, Ramalhete, Mulhovo, Duarte, & Ferreira, 2012; Pereira et al., 2016; Ramalhete et al., 2010), bioassay-guided fractionation of the methanol extract of the root barks of *C. kirkii* led to the isolation of several compounds with different scaffolds. Among them, *C*-benzylated flavanones, namely, dichamanetin and the α,β-unsaturated lactone (−)-cleistenolide, have shown relevant antibacterial activity against Gram-positive bacteria, including drug-resistant strains (Pereira et al., 2016). Moreover, in the combination with β-lactam antibiotics and vancomycin, polycarpol bearing a triterpenic scaffold exhibited a strong synergistic effect against Gram-positive resistant strains. In this study, polycarpol (**1**) and *C*-benzylated flavanones **2**–**4** and a heptane derivative (**5**) were further investigated for their ability as both EP and QS inhibitors of the efflux systems of Gram-negative and Gram-positive bacteria. Furthermore, the ability of compounds **2** and **4** to potentiate the effect of tetracycline and the fluoroquinolone antibiotic ciprofloxacin on methicillin-susceptible and methicillin-resistant *S. aureus* (MRSA) strains was also described.

2 | MATERIALS AND METHODS

2.1 | Compounds tested

Triterpene polycarpol (**1**), *C*-benzylated flavanones chamanetin (**2**), isochamanetin (**3**), dichamanetin (**4**), and the heptane derivative acetylmelodorinol (**5**; Figure 1) were isolated from the methanol extract of the root barks of *C. kirkii*, as it has previously been described (Pereira et al., 2016). The purity of all the compounds was higher than 95% based on high-performance liquid chromatography analysis and nuclear magnetic resonance spectroscopy. The compounds were dissolved in dimethyl sulfoxide (DMSO).

2.2 | Reagents and media

Promethazine (PMZ; EGIS), ethidium bromide (EB), verapamil, tetracycline-hydrochloride (TET), ciprofloxacin-hydrochloride (CIP), and Luria–Bertani (LB) broth, and LB agar were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The modified LB medium (LB*) was prepared from yeast extract 5 g/L, tryptone 10 g/L, NaCl 10 g/L, K_2HPO_4 1 g/L, $MgSO_4 \times 7H_2O$ 0.3 g/L, and FeNaEDTA

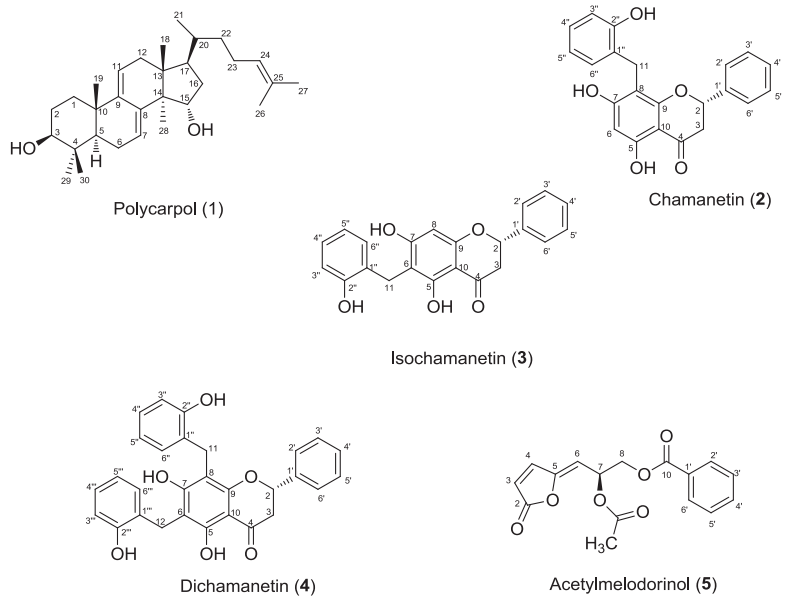

FIGURE 1 Chemical structures of compounds **1**–**5**

36 mg/L. In case of modified LB* agar, the LB* medium was supplemented with agar 20 g/L (Difco). pH was adjusted to 7.2. Tryptic soy broth (TSB), tryptic soy agar, and Mueller–Hinton (MH) broth were purchased from Scharlau Chemie S. A. (Barcelona, Spain).

2.3 | Bacterial strains

Compounds were evaluated against the Gram-negative wild-type *Escherichia coli* K-12 AG100 strain [argE3 thi-1 rpsL xyl mtl Δ(gal-uvrB) supE44], expressing the AcrAB–TolC EP at its basal level and its AcrAB–TolC-deleted mutant *E. coli* AG100 A strain. These strains were kindly provided by Prof. Dr. Hiroshi Nikaido (Department of Molecular and Cell Biology and Chemistry, University of California, Berkeley, CA, USA).

The compounds were further evaluated against two Gram-positive strains, *S. aureus* American Type Culture Collection (ATCC) 25923, used as the methicillin-susceptible reference strain, and the methicillin and ofloxacin-resistant *S. aureus* 272123 clinical isolate, which was kindly provided by Prof. Dr. Leonard Amaral (Institute of Hygiene and Tropical Medicine, Lisbon, Portugal).

For QS tests, the following strains were used: *Chromobacterium violaceum* 026 (CV026) as sensor strain and *Enterobacter cloaceae* 31298 as AHL producer strain (a clinical isolate from a wound). When *C. violaceum* reaches a high cell density, it produces a purple pigment, namely, violacein (Ballantine, Beer, Crutchley, Dodd, & Palmer, 1958).

2.4 | Determination of minimum inhibitory concentrations by microdilution method

The minimum inhibitory concentrations (MICs) of compounds were determined according to the Clinical and Laboratory Standard Institute guidelines (2017) in three independent assays. The solvent DMSO had no antibacterial effect.

2.5 | Interaction between antibiotics and compounds

The combined effect of compounds and antibiotics on the growth inhibition of *S. aureus* was evaluated by the checkerboard method. Twofold serial dilutions of antibiotics were prepared in MH broth on the horizontal rows of microtiter plate and then cross-diluted vertically by twofold serial dilutions of the compounds (Wolfart et al., 2006). For this assay, only the compounds with well-defined MIC values could be used. Consequently, the combination assays were carried out on methicillin-susceptible and MRSA strains. The dilutions of the antibiotics (TET or CIP) were made in a horizontal direction in 100 μl, and the dilutions of compounds were made vertically in the microtiter plate in 50 μl. After the dilution of an overnight culture, bacterial cells were resuspended in MH medium containing 1×10^4 cells and distributed into each well. The plates were incubated for 18 hr at 37 °C. The cell growth rate was determined after MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) staining, as described elsewhere (Wolfart et al., 2006). The combination index (CI) values at 90% growth inhibition (ED_{90}) were determined by using CompuSyn software to plot 4 or 5 data points for each ratio (www.combosyn.com, ComboSyn, Inc., Paramus, NJ, USA). CI values were calculated by means of the median-effect equation, where CI < 1, CI = 1, and CI > 1 represent synergism, an additive effect (or no interaction), and antagonism, respectively (Chou & Martin, 2005).

2.6 | Real-time accumulation assay by Roche LightCycler real-time thermocycler

The activity of compounds on the real-time accumulation of EB was assessed by the automated EB method (Viveiros et al., 2008) using a LightCycler real-time thermocycler (LightCycler 1.5, Roche, Indianapolis, IN, USA). Briefly, an aliquot of an overnight culture of the *S. aureus* strain in TSB medium was transferred to fresh TSB medium, and it was incubated until it reached an optical density (OD) of 0.6 at 600 nm. In case of *E. coli*, the medium used in the assay was LB broth; the preparation of the inoculum was similar to the one of *S. aureus*. The cells were washed with phosphate-buffered saline (PBS; pH 7.4) and centrifuged at 13,000 × *g* for 3 min, the pellets were resuspended in PBS (pH 7.4), and the OD was adjusted to 0.6 at 600 nm. The compounds were added individually at different concentrations at MIC/2, MIC/3, MIC/4, or MIC/5 (in double concentrated form) to the EB solution in PBS. The final concentration of EB was based on the MIC and the fluorescent signal produced by this amount of EB. In case of *S. aureus* strains, the concentration of EB was 0.5 μg/ml, for *E. coli* AG100 1 μg/ml, and in case of *E. coli* AG100 A, it was 0.25 μg/ml. Then, 10 μl of the EB solution containing the compound was transferred into standard glass capillary tubes of 20 μl maximum volume (Roche), and 10 μl of bacterial suspension (OD of 0.6 at 600 nm) was added to the capillaries. The capillaries containing the samples were placed into the carousel (Roche), and the fluorescence was monitored at the FL-2 channel in every minute on a real-time basis.

From the real-time data, the activity of the compound, namely, the relative final fluorescence index (RFI) of the last time point (minute 30) of the EB accumulation assay, was calculated according to the following formula:

$$RFI = \frac{RF_{\text{treated}} - RF_{\text{untreated}}}{RF_{\text{untreated}}}$$

where RF_{treated} is the relative fluorescence at the last time point of EB retention curve in the presence of an inhibitor and $RF_{\text{untreated}}$ is the relative fluorescence at the last time point of the EB retention curve of the untreated control having the solvent control (DMSO). Verapamil was applied as positive control on Gram-positive strains, and PMZ was used on Gram-negative strains.

2.7 | Assay for quorum sensing inhibition

LB* was used for these experiments. The sensor strain *C. violaceum* 026 and the AHL producer strains *E. cloaceae* 31298 were inoculated as parallel lines and incubated at room temperature (20 °C) for 24–48 hr. QS inhibition was monitored by the agar diffusion method. Filter paper discs (7.0 mm in diameter) were impregnated with 10 μl of stock solutions (10 mM) of the compounds in DMSO. The discs were placed between the parallel lines of the sensor and the AHL producer strains on the surface of the nutrient agar. The plates were incubated at room temperature for another 24–48 hr, and the interactions between the strains and compounds were evaluated for the

reduction in the size of the zone of pigment production and the zone of growth inhibition of the affected strains, in millimeters. PMZ was applied as positive control (Varga et al., 2011).

2.8 | Expression analyses of genes by real-time reverse transcriptase quantitative polymerase chain reaction

S. aureus ATCC 25923 and *S. aureus* 272123 strains were cultured in TSB broth and were incubated overnight at 37 °C with shaking. On the day of RNA isolation, the bacterial suspensions (OD of 0.6 at 600 nm) were transferred to 10-ml tubes in 3-ml aliquots, and 5 μM of compound **2** or 0.5 μM of compound **4** was added to the tubes, which were incubated at 37 °C. After 4 hr of culturing, the tubes were centrifuged at 12,000 × *g* for 2 min. Pellets were suspended in 100 μl Tris–EDTA buffer containing 1 mg/ml lysozyme by vigorous vortexing, and they were incubated at 37 °C for 10 min. The total RNA was isolated in an RNase-free environment using the NucleoSpin RNA kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. Purified RNA was stored in RNase-free water in nuclease-free collection tubes and was maintained at −20 °C until quantification was performed. The concentration of the extracted RNA templates was assessed by spectrophotometry at 260 nm. Expression of the EP genes *norA* and *mepA* was studied by reverse transcription of the total RNA. The data obtained for gene targets were normalized against the *S. aureus* 16S ribosomal RNA measured in the same sample. The primers (Couto, Costa, Viveiros, Martins, & Amaral, 2008) used in the assay were the following:

1. Sequence (5′-3′) of *norA* (246 bp)	TCGTCTTAGCGTTCGG TTTA (Fw)
	TCCAGTAACCATCGGC AATA (Rv)
2. Sequence (5′-3′) of *mepA* (198 bp)	TGCTGCTGCTCTGTTC TTTA (Fw)
	GCGAAGTTTCCATAAT GTGC (Rv)
3. Sequence (5′-3′) of *16S rRNS* (492 bp)	AGAGTTTGATCMTGGC TCAG (Fw)
	GWATTACCGCG GCKGCTG (Rv)

Real-time quantification of the RNA templates by real-time one-step reverse transcriptase quantitative polymerase chain reaction was performed in a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA), and the manufacturer's recommendations of the SensiFAST™ SYBR No-ROX One-Step Kit (Bioline GmbH, Luckenwalde, Germany) were strictly adhered to. Briefly, each well of the 96-well microtiter plates in a final volume of 20 μl contained 10 μl of the 2× SensiFAST™ SYBR No-ROX One-Step Mix, 0.2 μl reverse transcriptase, 0.4 μl RiboSafe RNase Inhibitor, 5.4 μl diethyl pyrocarbonate-treated water, 500 nM of each primer, and approximately 20 ng of the total RNA in RNAase-free water. Thermal cycling was initiated with a denaturation step of 5 min at 95 °C, followed by 40 cycles, each of 10 s at 95 °C, 30 s at 57 °C, and 20 s at 72 °C.

3 | RESULTS

3.1 | In vitro antibacterial activity of compounds

Compounds (**1**–**5**) were assessed for their antibacterial activity against methicillin-susceptible *S. aureus* ATCC 25923, and the methicillin- and ofloxacin-resistant *S. aureus* 272123 clinical isolate. Wild-type *E. coli* K-12 AG100 strain, and *E. coli* AG100 A strain, overexpressing and lacking the AcrAB–TolC EP system, respectively, were used as Gram-negative models. In addition, the antibacterial activity of the compounds was tested on QS strains *C. violaceum* and *E. cloaceae*.

Concerning the antibacterial effect of the compounds, chamanetin (**2**) and dichamanetin (**4**) had a potent antibacterial effect on the *S. aureus* strains. MIC value of compound **2** was 12.5 μM on reference *S. aureus*; however, the MIC of the methicillin- and ofloxacin-resistant strain was 25 μM. Compound **4** was the most effective flavanone because its MIC value on *S. aureus* ATCC 25923 was 0.8 μM; furthermore, on the methicillin-resistant strain, it was 1.56 μM.

The compounds had no antibacterial effect on the Gram-negative *E. coli* AG100, AG100 A, *C. violaceum*, and *E. cloaceae* strains.

3.2 | Combination effects of chamanetin (2) and dichamanetin (4) with antibiotics

The type of interaction between the antibacterial *C*-benzylated flavanones **2** and **4** and tetracycline and the fluoroquinolone antibiotic ciprofloxacin was evaluated on methicillin-susceptible (ATCC 25923) and MRSA strains by the checkerboard assay. The results are presented in Tables 1a and 1b as CI values. CI values <1 indicate a synergistic interaction between the compound and the antibiotic (Chou & Martin, 2005). As it can be observed, the combined effect of TET and compound **2** or **4** on *S. aureus* ATCC 25923 resulted in synergism. The most effective ratio of antibiotic and compound was 1:20

TABLE 1a Combination assays on *Staphylococcus aureus* ATCC 25923 strain

Staphylococcus aureus ATCC 25923				
Combination	**Best ratio**	**CI at ED_{90}**	**SD (+/−)**	**Interaction**
Tetracycline + chamanetin (**2**)	1:20	0.63786	0.13419	Synergism
Tetracycline + dichamanetin (**4**)	1:1	0.42093	0.10354	Synergism
Ciprofloxacin + chamanetin (**2**)	1.3:12.5	0.81577	0.23974	Slight synergism
Ciprofloxacin + dichamanetin (**4**)	1.3:1	0.68615	0.27953	Synergism

Note. Starting concentration of tetracycline: 5.2 μM; ciprofloxacin: 2.6 μM; and compound **2**: 25 μM and compound **4**: 2 μM. ATCC = American Type Culture Collection; CI = combination index.

TABLE 1b Combination assays on MRSA 272123 strain

Staphylococcus aureus 272123				
Combination	**Best ratio**	**CI at ED_{90}**	**SD (+/−)**	**Interaction**
Tetracycline + chamanetin (**2**)	6:25	1.4595	0.29390	Antagonism
Tetracycline + dichamanetin (**4**)	100:1	0.59402	0.34521	Synergism
Ciprofloxacin + chamanetin (**2**)	13:8	0.85030	0.21648	Slight synergism
Ciprofloxacin + dichamanetin (**4**)	32:1	1.35064	0.24946	Moderate antagonism

Note. Starting concentration of tetracycline: 100 μM; ciprofloxacin: 64 μM; and compound **2**: 50 μM and compound **4**: 3 μM. Ratio: antibiotic and tested compound (μM). CI < 1, CI = 1, and CI > 1 represent synergism, an additive effect (or no interaction), and antagonism, respectively. CI = combination index; MRSA = methicillin-resistant *Staphylococcus aureus*.

and 1:1, respectively. Similarly, CIP also acted synergistically with compounds **2** and **4**, being the most active ratio of antibiotic and compound 1.3:12.5 and 1.3:1, respectively (Table 1a).

Against the MRSA strain, compound **2** showed antagonistic effect with TET and slight synergism with CIP. The interactions of compound **4** with TET and CIP on the MRSA strain were synergism and moderate antagonism, respectively (Table 1b).

3.3 | Efflux pump-inhibiting activity

The EB accumulation assay provides information about the intracellular accumulation of the general EP substrate EB. A potential EPI increases the fluorescence level of EB because of its accumulation within the bacterial cell. The EP-inhibiting activity of the compounds was compared on the basis of the RFI of the real-time accumulation curves in Gram-positive and Gram-negative strains (Figure 2a). In case of real-time EB accumulation by the LightCycler thermocycler, the amount of EB accumulated by cells is higher if the difference between $RF_{treated}$ and $RF_{untreated}$ is greater; therefore, the degree of inhibition of the EP system by the compound becomes greater.

As shown in Figure 2a, compounds **2**, **3**, and **5** had EP-inhibiting activity compared with verapamil (RFI: 0.29) on the *S. aureus* ATCC 25923 strain, and the most active compound was compound **2**. However, compounds **1–5** had no EPI activity on the MRSA strain at the concentrations applied in the assay.

Concerning the inhibitory activity on Gram-negatives, triterpene polycarpol (**1**) and acetylmelodorinol (**5**) compared with the PMZ (RFI: 0.15) could inhibit the AcrAB–TolC system of *E. coli* AG100. Compound **1** proved to be the most effective EPI (Figure 2a). On the basis of the real-time accumulation data, compounds **1–5** had no effect on the *E. coli* AG100 A strain lacking the AcrAB–TolC pump.

3.4 | Anti-quorum sensing activity

The QS inhibition activity of compounds was defined measuring the colorless zone around the disc on *C. violaceum* as described previously (Varga et al., 2011). Compounds **1**, **2**, **4**, and **5** were able to inhibit effectively the QS between CV026 and *E. cloaceae* compared with the positive control PMZ (Table 2).

3.5 | Relative expression of efflux pump genes

In order to evaluate the effect of compounds on the relative expression of EP genes in both *S. aureus* strains, the most effective compounds **2** and **4** were selected for gene expression studies. In the real-time quantitative RT-PCR assay, the genes of NorA and MepA transporters were investigated. As shown in Figure 2bA, compound **2** at 5 μM significantly up-regulated the expression of *norA* and *mepA* genes after 4 hr of exposure in the MRSA strain. Compound **4** at 0.5 μM also significantly up-regulated both EP genes after 4 hr of exposure in the MRSA as presented in Figure 2bB. In the *S. aureus* ATCC strain, the expression level of the *mepA* gene was not influenced; nevertheless, the *norA* gene was significantly up-regulated by compounds **2** (19.84-fold increase) at 5 μM and **4** (2.39-fold increase) at 0.5 μM (data not shown).

4 | DISCUSSION

Natural compounds isolated from plants represent a valuable source of new antibacterial agents. When analyzing the results, the potent antibacterial activity found for dichamanetin (**4**) and also the significant activity of chamanetin (**2**) corroborated our previous studies on this set of rare *C*-benzylated flavanones (Pereira et al., 2016). The strongest activity of dichamanetin (**4**), when compared with chamanetin (**2**), seems to be due to its higher lipophilic character resulting from the extra benzyl group at C-6. In contrast to chamanetin (**2**), isochamanetin (**3**), differing only in the position of the benzyl group, was inactive at the concentration tested. Thus, besides the importance of lipophilicity, the presence of a benzyl moiety at C-8 appears to play a decisive role in the antibacterial activity of this type of compounds.

In the combination assays, chamanetin (**2**) and dichamanetin (**4**) had synergistic activity with tetracycline and ciprofloxacin on the *S. aureus* ATCC 25923 strain. Furthermore, on the MRSA strain, dichamanetin (**4**) and chamanetin (**2**), combined with tetracycline and ciprofloxacin, respectively, also showed synergism, which indicates that these compounds could be potential adjuvants in the therapy.

Besides having an antibacterial effect, both compounds (**2** and **4**) could inhibit the activity of EPs, they were QS inhibitors, and they significantly increased the expression of EP genes *norA* and *mepA* after 4 hr of exposure in the MRSA strain. Furthermore, the NorA and MepA pumps of the MRSA strain could not be inhibited because of the overexpression of these pumps. The change in gene expression could be due to the stress response against compounds **2** and **4** because these compounds as potential noxious agents for *S. aureus* had to be extruded from the cytoplasm as soon as possible. This stress response can be the explanation for the up-regulation of the EP genes after 4 hr. The difference between MRSA and ATCC is due to the overexpression

(a)

Compound	*E. coli* AG100			*S. aureus* ATCC 25923		
	MIC (μM)	Concentration (μM)	RFI	MIC (μM)	Concentration (μM)	RFI
1	>100	50	0.484	>100	25	-0.035
2	>100	50	-0.414	12.5	5	-0.267
3	>100	50	-0.032	>100	25	0.174
4	>100	50	-0.643	0.8	0.5	-0.151
5	>100	50	0.161	>100	25	0.139

(b)

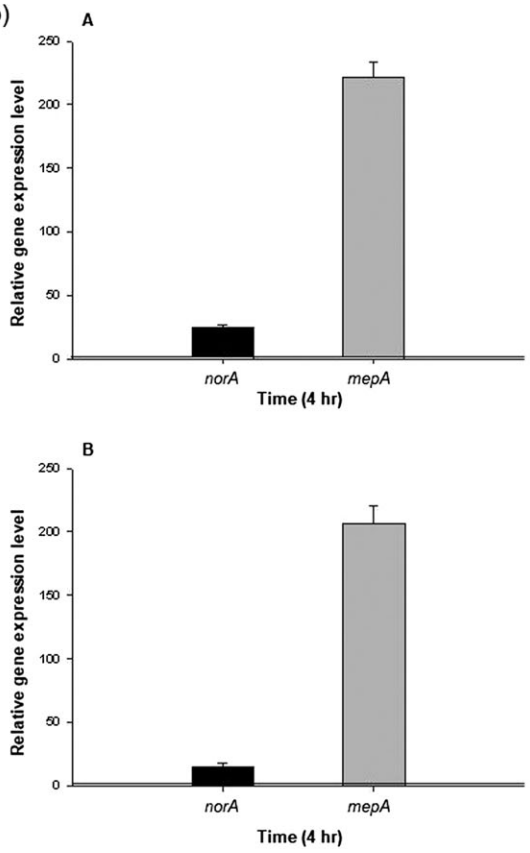

FIGURE 2 The minimum inhibitory concentration (MIC) and efflux pump modulating effect of compounds **1–5**. (a) Relative final fluorescence index (RFI) for the effect of compounds **1–5** on the AcrAB–TolC-expressing *Escherichia coli* AG100 and *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923 strains. (b) Relative gene expression levels of *norA* and *mepA* genes in the presence of (A) chamanetin (**2**) and (B) dichamanetin (**4**) in methicillin resistant *Staphylococcus aureus* (MRSA) 272123, after 4-hr exposure. The line denotes the threshold value, which was set at a twofold increase in transcripts

TABLE 2 Inhibitory effects of compounds **1–5** on QS signal transmission

Compound	QS inhibition zone in mm
1	51
2	50
3	–
4	53
5	52
Promethazine	46

Note. Ten microliters of 10 mM stock solution was added into the filter paper discs (10 μM per disc), and the colorless zone around the disc measured on *Chromobacterium violaceum* was measured after incubation for 24–48 hr at room temperature. QS = quorum sensing.

of the *mepA* gene in the resistant strain and it has low expression level in the ATCC strain. Compounds **2** and **4** increased the expression of *mepA* gene in the MRSA, but in the ATCC strain, these compounds did not influence the expression level of *mepA*. It has been demonstrated by other studies that the overexpression of EPs confers a fitness cost for the organism, for example, a resistant isolate overexpressing EPs shows reduced production of virulence determinants. Usually the overexpression of pump genes is often related to global effects on bacterial physiology influencing virulence (Ledda et al., 2017).

In Gram-negatives, compounds **1–5** had no antibacterial activity. Polycarpol (**1**) was the most effective EPI, inhibiting the AcrAB–TolC transport system in the *E. coli* AG100 strain. The AcrAB–TolC system belongs to the resistance nodulation division transporters, utilizing the proton motive force to extrude toxic agents out of the bacterial

cell. Since compound **1** had no EPI effect on the AcrAB–TolC pump-deleted *E. coli* strain, it may have a direct EPI effect on the AcrAB–TolC transporter system. The effect of polycarpol could be due to its high lipophilicity (Pereira et al., 2016), which can increase the membrane permeability of bacteria.

In addition to flavanones **2** and **4**, polycarpol (**1**) and acetylmelodorinol (**5**) were also able to inhibit effectively the bacterial communication, suggesting that they could be applied as anti-QS agents.

5 | CONCLUSIONS

To summarize our findings, *C. kirkii* constituents, chamanetin (**2**) and dichamanetin (**4**), inhibited the growth of both *S. aureus* strains. The three flavanones tested (**2**–**4**) increased the accumulation of the EP substrate EB on sensitive *S. aureus* strain, and compounds **1**, **2**, **4**, and **5** prevented the cell-to-cell communication. Polycarpol (**1**) was an effective inhibitor of the AcrAB–TolC system of *E. coli*, which might be explained by its interference with membrane permeability and modulation of the function of the pump. Dichamanetin (**4**) and chamanetin (**2**) showed synergistic effect on MRSA and sensitive *S. aureus* strains implying that they could restore and increase the efficacy of antibiotics, especially in MDR infections. It can be assumed that the advantageous properties of *C. kirkii* constituents can influence the QS system and operation of EPs decreasing virulence and antibiotic resistance (Varga et al., 2011).

According to the results, the most active compounds, chamanetin (**2**) and dichamanetin (**4**), could be possible resistance modifiers because they possess antibacterial, EPI, and anti-QS properties; moreover, these compounds might be used as adjuvants in the antibiotic therapy because they may be able to reduce the bacterial virulence. Furthermore, they can potentiate the activity of antibiotics.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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